

The Human Bone Sialoprotein Gene Contains an NF-E1/YY1 Cis-Acting Sequence with Putative Regulatory Activity

J. M. Kerr, D. R. R. Hiscock, W. Grzesik, P. G. Robey, M. F. Young

Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892

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Abstract. Bone sialoprotein (BSP) is a noncollagenous matrix glycoprotein localized predominantly in mineralized tissues but also detected in extraskelatal sites undergoing focal mineralization. We have previously characterized the human BSP gene and have shown that the upstream sequence contains inverted TATA and CCAAT motifs at the expected locations from the transcriptional start site (J. M. Kerr et al. [13]) and a potential YY1 binding motif located within the first 30 bp of intron 1 of the human gene. Deletion analyses of the human BSP promoter/exon 1 sequence fused to a CAT reporter gene indicate that CCAAT enhances basal transcription of BSP in transiently transfected rat UMR106-01 BSP osteosarcoma and rat skin fibroblasts. Though this enhancing activity was lost with inclusion of 68 bp of intron containing a YY1 motif in these constructs, reporter activity in the UMR106-01-BSP cells was elevated four- to seven-fold relative to that of rat fibroblasts. Gel electrophoretic mobility shift, UV-crosslinking, and south-western experiments indicate that YY1 is present only in the extracts of nuclei isolated from the UMR cells and may contribute to the elevated transcriptional activity of the human BSP promoter construct in UMR106-01-BSP.

Key words: Bone sialoprotein — UMR106-01 BSP — YY1 motif — CCAAT — TATA motif.

Bone sialoprotein (BSP) is a sialic, acid-rich, extracellular matrix protein which purportedly binds to the integrin $\alpha_v\beta_3$, and is highly conserved among animal species [1–7]. In humans [8, 9] and rats [10, 11], expression of BSP is primarily localized to cells of mineralized tissues including mature osteoblasts, osteoclasts, and hypertrophic chondrocytes. Two sites of extraskelatal expression have been identified in trophoblasts [8] and breast tumor cells [12], both of which can undergo focal mineralization.

The tissue-restricted and maturational stage-specific expression of this gene indicate that it is under tight regulatory control. The human [13, 14] and rat [15] genes have been cloned and partially characterized. Computer analyses of the upstream region of the gene as well as intronic sequences indicate numerous potential transcription factor binding sites. The canonical TATA and CCAAT boxes are observed at the correct distances from the transcriptional start site although both are inverted in orientation. A binding site for the zinc finger transcription factor, Yin Yang-1 (YY1), is located within the first 30 bp of intron 1 in the

human gene. YY1 can activate or inhibit transcription of a number of genes [reviewed in 16] or serve as an initiator of transcription [17]. We investigated the role of the inverted CCAAT and TATA binding sites, in the presence and absence of intronic sequence containing a YY1 motif, on transcriptional activity in a rat osteosarcoma line (UMR106-01-BSP) that constitutively expresses high levels of BSP, and in a rat fibroblast cell line that does not express BSP.

Materials and Methods

Cell Culture

Rat skin fibroblasts (RSF, ATCC CRL1213) and the rat osteogenic cell line, UMR106-01-BSP, were used in this study. The latter cell type was previously characterized in detail by Midura et al. [18] who showed that the cells synthesize and secrete relatively large amounts of a sulfated glycoprotein that was identified as BSP. The line was a kind gift from Dr. Midura and subsequently has been renamed UMR106-01-BSP. Both cell types were cultured in EMEM supplemented with 1 X Earle's salts containing glutamine (MediaTech), 1 X nonessential amino acids, 20 mM HEPES, and 10% fetal bovine serum (GIBCO). All cells were plated at an initial density of 20,000 cells/cm² in 60 mm tissue culture dishes and grown to 80–90% confluency for transient transfections, nuclear extract preparations, and RNA isolation.

Transient Transfections

Three promoter constructs, -319, -43, and -17, were generated by engineering a *Hind*III site into the upstream gene-specific (-319: 5'-agtgaagctgtgtataatta-3'; -43: 5'-tggtgttgaagcttaagaagag-3'; -17: 5'-tagccagcaagcttaagtgaatga-3') primers and engineering a *Bam*HI site into the downstream (+79: 5'-aaatcgatcctctggca-3'; +148: 5'-aaaattcgatccaagttcaga-3') gene-specific primers. Human BSP genomic fragments subcloned into Bluescript (Stratagene) were used as templates for polymerase chain reaction (PCR) amplification. The PCR products were restriction digested and ligated to the *Hind*III/*Bam*HI sites of the chloramphenicol acetyltransferase (CAT) reporter plasmid CB106 [19]. The luciferase plasmid was used to normalize for transfection efficiency. Both reporter genes were purified by a double CsCl gradient banding technique [20]. Twenty micrograms of the CAT construct and 2 μ g of luciferase plasmid were transiently co-transfected into subconfluent cells by the calcium phosphate transfection method [20].

Cells were rinsed and supplemented with fresh medium after 24 hours, and harvested in 100 μ l of 0.25 M Tris-HCl, pH 8.0 after an additional 24-hour period. Cells were lysed by three freeze-thaw cycles, centrifuged, and the supernatant was stored at -70°C until use. Ten microliter aliquots of cell lysate were assayed for luciferase activity as previously described [21] using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). CAT activity was assayed using 30- μ l aliquots of cell lysates (previously heated for 10 minutes at 65°C) in a total reaction

volume of 125 μ l of 0.25 M Tris-HCl, pH 8.0, containing 25 μ g n-butyrylCoA and 5 μ l [14 C]chloramphenicol (Amersham, Arlington Heights, IL). After a 24-hour incubation period at 37°C, the butyrylated products were extracted and analyzed by the xylene extraction/liquid scintillation counting method (Promega Technical Bulletin #84). Transfections were performed in triplicate and replicated a minimum of two times to ensure reproducibility. The data, reported as CAT/luciferase, were analyzed by analysis of variance, and the Student Newman Keul's means comparison procedure was used to determine significant differences among the means [23].

Protein-DNA Interactions

Crude nuclear extracts were obtained as previously described [20] and stored at -70°C until use. Protein concentrations were determined by the BCA Micro-Protein assay (Pierce) using bovine serum albumin as the standard. Deoxyoligonucleotides were synthesized using an Expedite Nucleic Acid Synthesis System (Millipore Corporation, Rockford, IL). The double-stranded deoxyoligonucleotide BSP-YY1 containing a potential YY1 binding site (underlined) and flanking sequence (5'-caacttcattatcataaaattaga-3') was end-labeled with [γ - 32 P]dATP (NEN) using T4 polynucleotide kinase (Promega, Madison, WI).

For gel shift analyses, 15 μ g nuclear protein or 1 μ g human recombinant YY1 protein [23] were incubated with 20,000 cpm of labeled BSP-YY1 in gel shift buffer (Stratagene) containing 1 μ g of poly(dI-dC) for 20 minutes at room temperature. The DNA-protein complexes were electrophoresed through a 6% polyacrylamide gel. The dried gel was subjected to autoradiography overnight at -70°C. Supershift experiments were conducted in a similar way except that 4 μ g of a mouse polyclonal anti-human YY1 antibody [23] was added for an additional 30 minutes on ice prior to electrophoresis.

For UV crosslinking experiments, incubations were performed as described with the gel shift assays followed by exposure to short-wave ultraviolet (UV) light (Fotodyne, Inc., New Berlin, WI) for 30 minutes. Specific (unlabeled BSP-YY1) and nonspecific (AP-2, Stratagene, La Jolla, CA) competitor deoxyoligonucleotides were added at 100 \times concentration relative to the end-labeled BSP-YY1. After crosslinking, 1 μ g proteinase K (Boehringer Mannheim) was added to a control sample for 5 minutes at 37°C. Samples were electrophoresed through a 4–20% SDS polyacrylamide gel. The dried gel was subjected to autoradiography at -70°C overnight.

Southwestern analyses were performed as previously described [24]. Briefly, aliquots with 10 μ g of nuclear protein were electrophoresed through a 4–20% SDS polyacrylamide gel and transferred to nitrocellulose by electroblotting according to the manufacturer's directions (MilliBlot-Graphite Electroblotter Systems, Millipore, Bedford, MA). The membrane was incubated in binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.02% BSA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 30 mg/ml poly(dI-dC)) for 30 minutes and subsequently washed three times over a period of 30 minutes in wash buffer [binding buffer without poly(dI-dC)]. The membrane was then incubated with end-labeled BSP-YY1 deoxyoligonucleotide in binding buffer containing 1 μ g/ml poly(dI-dC) for 60 minutes followed by extensive washing with the same buffer and subjected to autoradiography for 48 hours at -70°C.

Northern and Reverse Transcription-PCR Analyses

Total RNA was extracted from UMR106-01 BSP and RSF cultures using RNA Stat-60 (Tel-Test, Inc., Friendswood, TX). For northern analyses, 10 μ g and 20 μ g of total RNA from UMR106-01 BSP and RSF cells, respectively, were electrophoresed through a 1.2% agarose-formaldehyde gel and transferred to a nitrocellulose membrane for hybridization to a random primed (Prime II, Stratagene) full-length [α - 32 P]dCTP-labeled rat BSP cDNA probe.

The membrane was subjected to autoradiography overnight at -70°C in the presence of an intensifying screen.

Because the rat YY1 sequence has not been reported to date, human YY1 and murine YY1 sequences (GenBank Accession # M76541 and # L13968 for human and mouse, respectively) were aligned by the Bestfit program (GCG) to identify conserved upstream (5'-atttgcgcacacatgtgc-3') and downstream (5'-tgtaagcaacaggtgagcttc-3') sequences for generation of a 500 bp reverse transcribed-PCR product for southern analysis. Reverse transcription-PCR (GeneAmp RNA PCR Kit, Perkin Elmer, Foster City, CA) was performed according to the manufacturer's specifications using 2 μ g of total RNA isolated from UMR106-01 BSP or RSF cell cultures. The products were electrophoresed through a 1.5% agarose gel and visualized by ethidium bromide staining for size estimation. The DNA was transferred to a nitrocellulose membrane and hybridized with a human/mouse conserved YY1 [γ - 32 P] end-labeled deoxyoligonucleotide (5'-gggaataaatatgcctctccttgc-3') located within the primer pair used to generate the PCR product. The membrane was subjected to autoradiography overnight at room temperature in the presence of an intensifying screen.

Results and Discussion

Comparison of the Human and Rat BSP Genes

A high degree of conservation of the BSP gene exists among a number of species, particularly within the coding regions for the Arg-Gly-Asp cell-binding domain, acidic amino acid stretches, and tyrosine-rich regions. A comparison of the previously reported 350 bp of upstream, noncoding exon 1 and the first 200 bp of intron 1 sequence of the human [13, 14] and rat [15] BSP genes indicates a high degree of similarity (86%) between the two species as well (Fig. 1). Analyses of these regions for transcription factor binding sites revealed conservation of the inverted TATA and CCAAT motifs at the expected location from the transcriptional start site, suggesting that these regions are important in the transcriptional regulation of BSP. Further evidence that the inverted motifs may be important for transcription initiation was derived from primer extension analyses, using human [13] and rat [15] RNA obtained from osteoblastic cells, where one major 5' terminus was identified downstream of the inverted TATA. We therefore initiated transient transfection experiments to determine whether the 5' flanking region, the inverted CCAAT and TATA motifs, and partial intronic sequence of the human gene were required for transcriptional activation. Human BSP constructs fused to the CAT reporter gene were transiently transfected into UMR106-01 BSP cells which constitutively express abundant BSP message (Fig. 2). RSF cells, which do not synthesize the BSP message (Fig. 2), were used as a control for the transient transfection experiments. It should be noted that these studies were also performed using normal human trabecular bone cell cultures, however, due to the low transfectability and the lack of BSP expression under the conditions needed for transfections, these cells could not be used.

Deletional Analysis of the 5' Flanking Sequence of the Human BSP Gene

5' deletion constructs were generated by PCR using the human gene as a template. A schematic of the constructs generated for transient transfections is shown in Figure 3A. Construct -319/+79 contained 319 bp of upstream sequence from the transcription start site and exon 1. Deletion constructs 43/+79 and -17/+79 removed upstream sequence through the inverted CCAAT and TATA motifs, respectively. A second set of constructs as generated to include 68

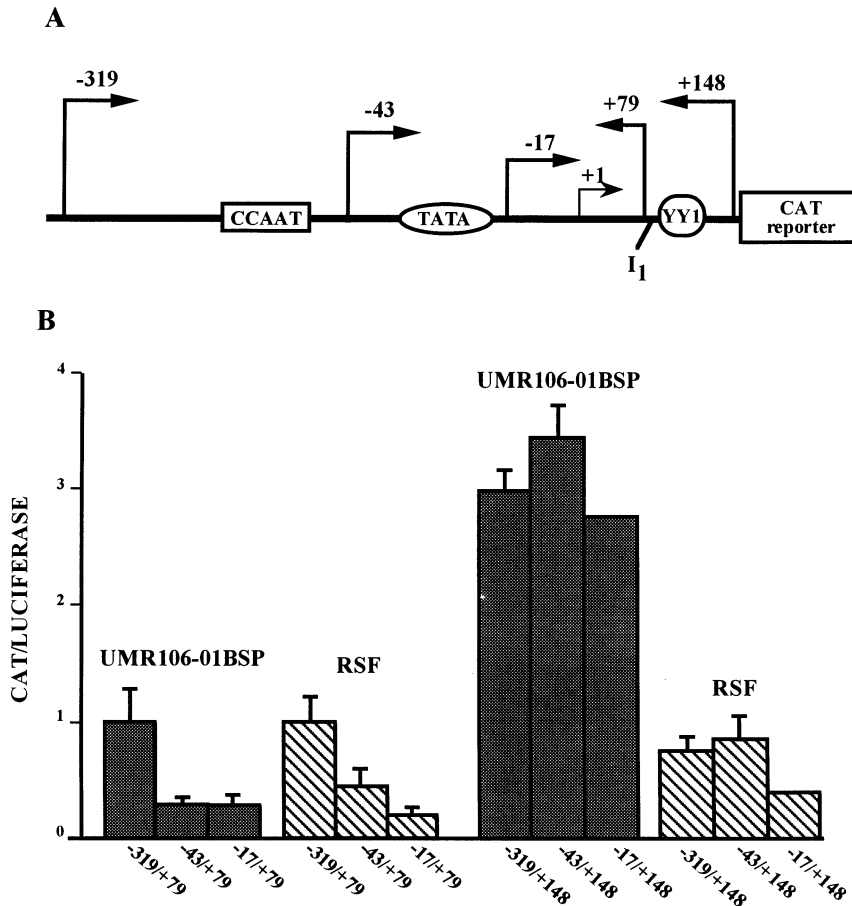


Fig. 3. Schematic drawing of human BSP 5' and intronic CAT reporter constructs, and transient transfection analyses in UMR106-01 BSP and RSF cells. **(A)** Numbers indicate the 5' and 3' boundaries of the constructs relative to the transcriptional start site (+1). The relative positions of the inverted CCAAT and TATA motifs and a potential YY1 binding site are shown. The position of I₁ indicates the exon/intron boundary. **(B)** Reporter activity measured as cat/luciferase (\pm SD) for transient transfection analyses are shown in the bar graph. UMR106-01 BSP and RSF cultures were transiently transfected with 20 μ g of human BSP promoter deletion constructs in the absence (+79) or presence (+148) of intronic sequence containing a YY1 motif, and 2 μ g of luciferase plasmid.

Extension of the 3' end of the promoter construct to include 68 bp of intron 1 resulted in a three- to seven-fold increase in reporter activity in UMR106-01 BSP compared with RSF cultures ($P \leq 0.01$). Addition of the intronic sequence, however, resulted in a loss of the enhancing effect of the 5' sequence through the CCAAT motif in UMR106-01 BSP and RSF cultures (Fig. 3B). The reason for the loss of enhancing activity is unknown, although there is widening evidence that DNA sequence downstream of the promoter region may convey information via a conformational mechanism or through direct DNA-protein interactions. We analyzed the intronic sequence for potential cis-acting elements that may contribute to the cell-specific increase in reporter activity observed in UMR106-01 BSP cells, using the transcription factor database (GCG). AYY1 binding motif (CATTATCAT), similar to an erythroid-specific protein binding motif, NF-E1 ((A/C)Py(T/A)ATC(A/T)Py) located in the human β -globin gene [28] and a motif in the GM-CSF core promoter (CATT(A/T)) [29]. This protein, renamed YY1, is a zinc-finger transcription factor that is highly conserved between human and mouse at the nucleic (95% identity) and protein (99% identity) levels and has been shown to both activate and suppress a number of cellular and viral genes [16]. The multifunctionality of this transcription factor may be attributed to two domains: the activation domain localized to the amino terminus, and the repression domain embedded in the zinc finger region near the carboxy terminus [30]. From a comparison of YY1 binding sites of a number of genes regulated by YY1, there appear to be two consensus sequences, a 15 bp activator sequence and an 11

bp repressor sequence [16]. However, examination of published reports of YY1 binding sites suggest that YY1 can tolerate variability in sequence flanking a 3 bp core motif, 5'-CAT-3' [31, 32]. We wished to determine if the YY1 transcription factor was responsible for the elevated reporter activity observed in UMR106-01 BSP cultures. To date, the presence of YY1 has not been demonstrated in UMR106-01 BSP and RSF cells. Therefore, we undertook experiments to detect expression of YY1 message, and to establish DNA binding activity in these cells.

Detection of YY1 Message in UMR106-01 BSP

RNA was isolated from UMR106-01 BSP and RSF cultures to detect YY1 message by reverse transcription-PCR analysis. Since the rat YY1 sequence has not been published, conserved sequences of the human and murine YY1 cDNAs were used to design a primer pair which would generate a 500 bp product by RT-PCR. Ethidium bromide staining of the PCR products showed a fragment of the expected size only in UMR106-01 BSP (Fig. 4, bottom panel). We confirmed that the PCR product was YY1 by southern analysis using an end-labeled conserved human/murine deoxyoligonucleotide (internal to the primer pair) as a probe. As with the ethidium bromide staining, a signal was only observed in the UMR106-01 BSP cells (top panel). The size of the PCR product was estimated from calculating its relative mobility compared with a broad range of molecular weight markers (λ Hind III/ ϕ X 174-Hae III digest). Partial

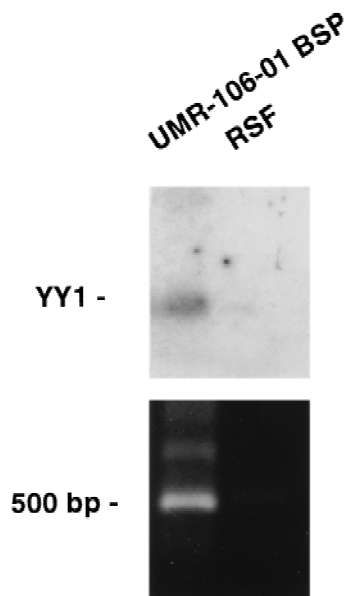


Fig. 4. Detection of YY1 message in UMR106-01 BSP and RSF by reverse transcription-PCR amplification. Aliquots with 2 µg of total RNA were reverse transcribed and amplified by PCR using primers homologous to the human and murine YY1 cDNA sequences. A southern blot of the products was probed with an internal probe homologous to both species (upper panel). The bottom panel represents ethidium bromide staining of 20 µl of PCR products electrophoresed through a 1.5% agarose gel.

DNA sequence of the PCR product confirmed that the product corresponded to the rat form of YY1.

Binding of a Nuclear Protein to a YY1 Motif

Gel shift analyses (Fig. 5) were undertaken to establish whether the BSP-YY1 site was capable of binding YY1. A single shifted complex was observed using UMR106-01 BSP (lane a) and RSF (lane b) nuclear extracts. Incubation of purified human recombinant YY1 with BSP-YY1 resulted in a single shift with a slower migration (lane c) than that of the rat cells. Confirmation that the shift was due to YY1 was determined by incubating the DNA/protein complex with an antibody to human recombinant YY1. Electrophoretic mobility shift analyses revealed a supershifted complex with nuclear extracts obtained from UMR106-01 BSP (lane d) and an attenuated signal with the human recombinant YY1 protein (lane f), which has been previously reported [24, 33, 34]. The RSF complex was neither supershifted nor ablated (lane e) and suggests that protein(s) other than YY1 can bind to the BSP-YY1 motif.

Size estimation of the protein was performed by UV crosslinking of the UMR106-01 BSP-derived nuclear factors with the end-labeled BSP-YY1 deoxyoligonucleotide (Fig. 6). A broad signal around 68 Kd in the absence (lane c) or presence (lane e) of a nonspecific competitor, AP-2, was observed. Addition of a specific inhibitor, BSP-YY1, was effective in competing with labeled BSP-YY1 (lane d). No complex was apparent in the absence of UV crosslinking (lane b) or when the crosslinked product was treated with proteinase K (lane f), indicating that the complex contained protein. Further confirmation of the protein size was obtained by southwestern analysis (Fig. 7). BSP-YY1 binding

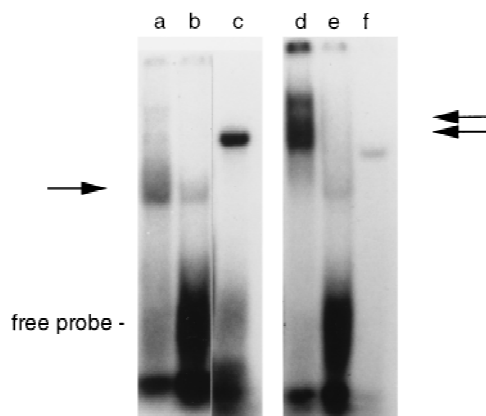


Fig. 5. Gel shift analyses of nuclear extracts isolated from UMR106-01 BSP and RSF cells using the BSP-YY1 probe. Aliquots of 15 µg of nuclear protein isolated from UMR106-01 BSP (lanes a and d) and RSF (lanes b and e) and 1 µg of human recombinant YY1 protein (lanes c and f) were incubated with the BSP-YY1 motif in the absence (lanes a,b,c) or presence (lanes d,e,f) of a human YY1 antibody. The shifted complex is indicated with a single arrow (in lane a it is intense and in lane b less intense). The supershifted complex is indicated by the double arrow shown in lane d as a very intense band higher than the same gel shift without the YY1 antibody.

	a	b	c	d	e	f
YY1 probe	+	+	+	+	+	+
nuclear protein	-	+	+	+	+	+
UV cross-linked	+	+	+	+	+	+
YY1 competitor	-	-	-	+	-	-
AP-2 competitor	-	-	-	-	+	-
proteinase K	-	-	-	-	-	+

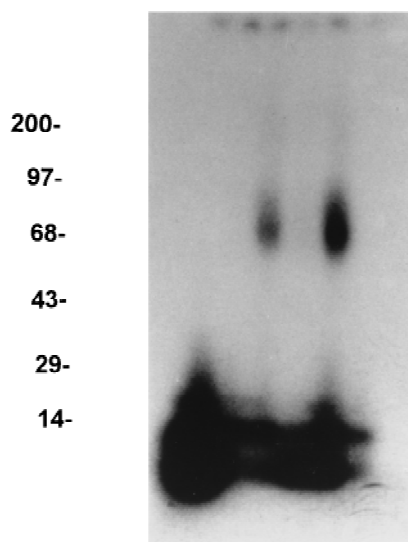


Fig. 6. Size estimation of the nuclear protein complexed to the BSP-YY1 deoxyoligonucleotide by UV crosslinking. An end-labeled BSP-YY1 probe (lanes a-f) was incubated with 15 µg of UMR106-01 BSP nuclear extract in the presence (lane d) or absence (lane b) of 100 X specific (BSP-YY1) or nonspecific (AP-2, lane e) competitors and crosslinked as described in Methods. Proteinase K (lane f) was added 5 minutes prior to electrophoresis through a 4–20% SDS polyacrylamide gel. The membrane was subjected to autoradiography for 24 hours at -70°C . The relative size of the crosslinked product was estimated by comparison to the migration of molecular weight protein standards (see left of the panel) run on the same gel.

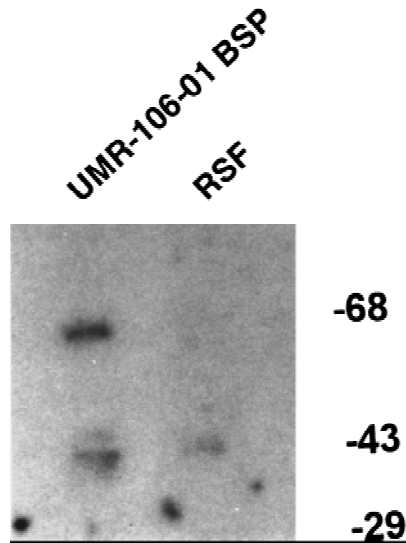


Fig. 7. Southwestern analyses of UMR106-01 BSP and RSF nuclear extracts. Nuclear extracts (15 μ g) were electrophoresed through a 4–20% SDS polyacrylamide gel, transferred to a nitrocellulose membrane by electroblotting, and probed with the end-labeled BSP-YY1 probe as described in Methods. The membrane was subjected to autoradiography for 72 hours at -70°C . The size of the protein that had affinity and bound the radioactive YY1 oligonucleotide was estimated from molecular weight protein standards run on the same gel (see right of gel).

activity with UMR106-01 BSP nuclear extracts revealed a signal at the expected size of approximately 68 Kd, as well as a signal with a lower molecular weight species. Complexation with this smaller molecular weight species was also observed using RSF nuclear extracts. Since a supershift was not apparent with RSF nuclear extracts, the smaller molecular weight species is probably not YY1. This unidentified smaller species has been previously noted in southwestern analyses conducted with a YY1 motif located in the site IV regulatory region of the histone 4 gene, and nuclear extracts obtained from the osteosarcoma line, ROS17/2.8 [35]. This smaller species was also detected by two-dimensional gel electrophoresis with a YY1 motif located in the human LINE-1 element and nuclear extracts obtained from Jurkat cells [24]. The size heterogeneity observed in both the UV crosslinking and southwestern analyses, therefore, may represent other nuclear factors capable of complexing with the YY1 motif [24, 27] although differential phosphorylation [25], and proteolytic degradation [37–39] may have also contributed to the size heterogeneity in the UMR106-01 BSP nuclear extracts.

The limited tissue distribution and temporal expression of bone sialoprotein [8–12, 40] indicates that this gene is subject to tight regulation. In osteoblastic cells, BSP is only observed when these cells have achieved a mature phenotype. It remains to be established why UMR106-01 BSP expresses abundant levels of BSP, however, this cell line may be representative of the mature osteoblast phenotype. Using this cell line, as well as a non-BSP producing fibroblast cell line for transient transfections of human BSP promoter constructs containing a YY1 motif, we were able to distinguish differential transcriptional activity. The demonstration of YY1 message and protein, and the ability of YY1 to bind to its recognition sequence in UMR106-01 BSP and not in RSF, led us to believe that YY1 is responsible for the

enhanced reporter activity observed in this osteosarcoma cell line. This type of differentiation-dependent regulatory activity by YY1 has been observed in primary myoblast cultures [40]. YY1 overexpression resulted in activation of *c-myc* and inhibition of skeletal α -actin expression (presumably through binding to its recognition sequence in the *c-myc* and skeletal α -actin promoter regions of the genes) thereby maintaining an undifferentiated myoblast phenotype. It is possible there may be a similar function for YY1 in osteoblastic cells and that the presence of YY1 and the capacity of this nuclear factor to bind to the BSP intragenic sequence, regulates the transcription of the human BSP gene. Definitive proof that YY1 contributes to the expression of this extracellular matrix protein in mature osteoblasts must await additional studies carried out *in vivo*.

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